PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
PBA/D088342PWO		
International application No.	International filing date *(day, month, year)*	(Earliest) Priority Date *(day, month, year)*
PCT/GB 00/ 00145	21/01/2000	21/01/1999
Applicant		
ADVANCED MEDICAL SOLUTIONS LIMITED et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   SUBSTRATE FOR CELL GROWTH

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/00145

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/00 A61L15/00 A61L27/00 C08L5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, EPO-Internal, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category ^	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 12228 A (THE REFENTS OF THE UNIVERSITY OF MICHIGAN) 26 March 1998 (1998-03-26) page 1, line 1 -page 3, line 5; claims; examples 1-3; table 1	1-31, 56-64
Y	page 14, line 2 - line 3 page 20, line 11 - line 18 page 25, line 10 -page 29, line 12	32-55
Y	WO 96 10106 A (INNOVATIVE TECHNOLOGIES LTD.) 4 April 1996 (1996-04-04) cited in the application the whole document	32-55

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

^ Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 September 2000	25/09/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ryckebosch, A

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	DATABASE WPI Section Ch, Week 199005 Derwent Publications Ltd., London, GB; Class A96, AN 1990-032925 XP002147316 & JP 01 309682 A (SANYO CHEM IND LTD), 14 December 1989 (1989-12-14) abstract -----	1,2,18, 28-30, 32,37,62

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/00145

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9812228 A	26-03-1998	AU 4493097 A	14-04-1998
		EP 0927196 A	07-07-1999
WO 9610106 A	04-04-1996	AU 3530695 A	19-04-1996
		EP 0783605 A	16-07-1997
		GB 2307687 A,B	04-06-1997
		JP 10506442 T	23-06-1998
		US 6080420 A	27-06-2000
JP 1309682 A	14-12-1989	NONE	

PA NT COOPERATION TREAT

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 12 September 2000 (12.09.00)	
International application No. PCT/GB00/00145	**Applicant's or agent's file reference** PBA/D088342PWO
International filing date (day/month/year) 21 January 2000 (21.01.00)	**Priority date** (day/month/year) 21 January 1999 (21.01.99)
Applicant HAMILTON, Douglas, William et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

17 August 2000 (17.08.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Olivia TEFY

Telephone No.: (41-22) 338.83.38

PA NT COOPERATION TREAT

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION RELATING TO PRIORITY CLAIM

(PCT Rules 26bis.1 and 26bis.2 and Administrative Instructions, Sections 402 and 409)

To:

ATKINSON, Peter, Birch
Marks & Clerk
Sussex House
83-85 Mosley Street
Manchester M2 3LG
ROYAUME-UNI

Date of mailing (day/month/year)
25 July 2000 (25.07.00)

Applicant's or agent's file reference
PBA/D088342PWO

IMPORTANT NOTIFICATION

International application No.
PCT/GB00/00145

International filing date (day/month/year)
21 January 2000 (21.01.00)

Applicant
ADVANCED MEDICAL SOLUTIONS LIMITED et al

The applicant is hereby **notified** of the following in respect of the priority claim(s) made in the international application.

1. ☐ **Correction of priority claim.** In accordance with the applicant's notice received on: , the following priority claim has been corrected to read as follows:

   ☐ even though the indication of the number of the earlier application is missing.
   ☐ even though the following indication in the priority claim is not the same as the corresponding indication appearing in the priority document:

2. ☒ **Addition of priority claim.** In accordance with the applicant's notice received on: 20 June 2000 (20.06.00), the following priority claim has been added:

   GB 21 January 1999 (21.01.99) 9901272-6

   ☐ even though the indication of the number of the earlier application is missing.
   ☐ even though the following indication in the priority claim is not the same as the corresponding indication appearing in the priority document:

3. ☐ As a **result of the correction and/or addition** of (a) priority claim(s) under items 1 and/or 2, the (earliest) priority date is:

4. ☐ **Priority claim considered not to have been made.**

   ☐ The applicant failed to respond to the Invitation under Rule 26bis.2(a) (Form PCT/IB/316) within the prescribed time limit.
   ☐ The applicant's notice was received after the expiration of the prescribed time limit under Rule 26bis.1(a).
   ☐ The applicant's notice failed to correct the priority claim so as to comply with the requirements of Rule 4.10.

   The applicant may, before the technical preparations for international publication have been completed and subject to the payment of a fee, request the International Bureau to publish, together with the international application, information concerning the priority claim. See Rule 26bis.2(c) and the PCT Applicant's Guide, Volume I, Annex B2(IB).

5. ☐ In case where **multiple priorities** have been claimed, the above item(s) relate to the following priority claim(s):

6. A copy of this notification has been sent to the receiving Office and
   ☒ to the International Searching Authority (where the international search report has not yet been issued).
   ☒ the designated Offices (which have already been notified of the receipt of the record copy).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

Lazar Joseph Panakal

Telephone No. (41-22) 338.83.38

Copy for the designated Office (DO/US)

PCT/GB00/00145

PATENT COOPERATION TREATY

PCT

NOTIFICATION RELATING TO PRIORITY CLAIM

(PCT Rules 26bis.1 and 26bis.2 and Administrative Instructions, Sections 402 and 409)

From the INTERNATIONAL BUREAU

To:

ATKINSON, Peter, Birch
Marks & Clerk
Sussex House
83-85 Mosley Street
Manchester M2 3LG
ROYAUME-UNI

Date of mailing (day/month/year) 25 July 2000 (25.07.00)	
Applicant's or agent's file reference PBA/D088342PWO	**IMPORTANT NOTIFICATION**
International application No. PCT/GB00/00145	International filing date (day/month/year) 21 January 2000 (21.01.00)
Applicant ADVANCED MEDICAL SOLUTIONS LIMITED et al	

The applicant is hereby **notified** of the following in respect of the priority claim(s) made in the international application.

1. ☐ **Correction of priority claim.** In accordance with the applicant's notice received on: , the following priority claim has been corrected to read as follows:

   ☐ even though the indication of the number of the earlier application is missing.
   ☐ even though the following indication in the priority claim is not the same as the corresponding indication appearing in the priority document:

2. ☒ **Addition of priority claim.** In accordance with the applicant's notice received on: 20 June 2000 (20.06.00), the following priority claim has been added:

   GB 17 February 1999 (17.02.99) 9903561-0

   ☐ even though the indication of the number of the earlier application is missing.
   ☐ even though the following indication in the priority claim is not the same as the corresponding indication appearing in the priority document:

3. ☐ As a **result of the correction and/or addition** of (a) priority claim(s) under items 1 and/or 2, the (earliest) priority date is:

4. ☐ **Priority claim considered not to have been made.**

   ☐ The applicant failed to respond to the Invitation under Rule 26bis.2(a) (Form PCT/IB/316) within the prescribed time limit.
   ☐ The applicant's notice was received after the expiration of the prescribed time limit under Rule 26bis.1(a).
   ☐ The applicant's notice failed to correct the priority claim so as to comply with the requirements of Rule 4.10.

   The applicant may, before the technical preparations for international publication have been completed and subject to the payment of a fee, request the International Bureau to publish, together with the international application, information concerning the priority claim. See Rule 26bis.2(c) and the PCT Applicant's Guide, Volume I, Annex B2(IB).

5. ☐ In case where **multiple priorities** have been claimed, the above item(s) relate to the following priority claim(s):

6. A copy of this notification has been sent to the receiving Office and
   ☒ to the International Searching Authority (where the international search report has not yet been issued).
   ☒ the designated Offices (which have already been notified of the receipt of the record copy).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Lazar Joseph Panakal
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

Form PCT/IB/318 (July 1998)

003428061

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

14

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PBA/D088342PWO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/00145	21/01/2000	21/01/1999
International Patent Classification (IPC) or national classification and IPC C12N5/00		
Applicant ADVANCED MEDICAL SOLUTIONS LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 7 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
17/08/2000	10.04.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Stolz, B Telephone No. +49 89 2399 8416

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/00145

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-18	as originally filed				

Claims, No.:

1-62	as received on	16/03/2001	with letter of	16/03/2001	

Drawings, sheets:

1/3-3/3	as originally filed				

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.

   ☐ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority in written form.

   ☐ furnished subsequently to this Authority in computer readable form.

   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:

   ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-62
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-62
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-62
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

1. Reasoned statement

1.1. The application describes cell growth substrates comprising a polysaccharide and a cell adhesion protein non-covalently attached thereto. The substrates can be spun into fibers or produced as sheets. They are useful for cell growth in vitro or for wound healing and tissue engineering.

1.2. The new set of claims is acceptable under Art. 34(2)(b) PCT.

1.3. Novelty (Art. 33(2) PCT)

Methods of producing polymeric substrates comprising polysaccharides and a non-covalently attached cell adhesion molecule have not been described in the cited prior art. The claimed methods and products are therefore novel.

1.4. Inventive step (Art. 33(3) PCT)

The closest prior art to the present application is WO98/12228 (D1), describing the use of a polymeric substrate such as alginate having covalently bound cell adhesion molecules. D1 described the limited usefulness of alginate hydrogels due to their lack of inherent cell adhesion (p. 5, lines 5-10). The solution to this problem provided by D1 is covalent attachment of cell adhesion molecules. The present application deals with the same technical problem but provides a different solution, i.e. non-covalent association of cell adhesion molecules with the polysaccharide substrate. Since the cited prior art does not mention non-covalent attachment of cell adhesion molecules, the claimed solution to the above mentioned technical problem cannot be said to be derivable from the prior art in an obvious way. The claims are therefore considered to be inventive.

2. Certain observations

2.1. Claim 18 lists suitable polysaccharides. Among these are also polyamino acids such as polylysine and the protein collagen (gelatin).

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 August 2000 (24.08.2000)

PCT

(10) International Publication Number
WO 00/49135 A3

(51) International Patent Classification[7]: C12N 5/00, A61L 15/00, 27/00, C08L 5/00

(21) International Application Number: PCT/GB00/00145

(22) International Filing Date: 21 January 2000 (21.01.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

9901272-6	21 January 1999 (21.01.1999)	GB
9903561-0	17 February 1999 (17.02.1999)	GB

(71) Applicant *(for all designated States except US)*: **ADVANCED MEDICAL SOLUTIONS LIMITED** [GB/GB]; Road Three, Winsford Industrial Estate, Winsford, Cheshire CW7 3PD (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **HAMILTON, Douglas, William** [GB/GB]; Heyes Park, Hartford, Northwich, Cheshire CW8 2AJ (GB). **IVES, Christopher, Louis** [GB/GB]; Rose Farm, Stonely Green, Nantwich, Cheshire CW5 8QA (GB). **MIDDLETON, Ian, Philip** [GB/GB]; 7 Kingsley Road, Boughton, Chester CH3 5RR (GB). **ROSSETTO, Chiara** [GB/GB]; 20 Vose Close, Hood Manor, Warrington, Cheshire WA5 1EW (GB).

(74) Agent: **ATKINSON, Peter, Birch**; Marks & Clerk, Sussex House, 83-85 Mosley Street, Manchester M2 3LG (GB).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

(88) Date of publication of the international search report:
14 December 2000

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/49135 A3

(54) Title: SUBSTRATE FOR CELL GROWTH

(57) Abstract: A substrate for cell growth comprises a polysaccharide and a cell adhesion protein provided at the surface of the substrate. The polysaccharide may for example be an alginate and the cell adhesion protein may be fibronectin, vitronectin or von Willebrand protein. The substrate may for example be a fibre and may be produced by extruding a solution containing dissolved polysaccharide and cell adhesion protein into a coagulation bath so that a substrate comprised of a basal layer of the polysaccharide and surface layer of cell adhesion protein is precipitated.

INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/GB 00/00145

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/00 A61L15/00 A61L27/00 C08L5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, EPO-Internal, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 12228 A (THE REFENTS OF THE UNIVERSITY OF MICHIGAN) 26 March 1998 (1998-03-26) page 1, line 1 -page 3, line 5; claims; examples 1-3; table 1	1-31, 56-64
Y	page 14, line 2 - line 3 page 20, line 11 - line 18 page 25, line 10 -page 29, line 12	32-55
Y	WO 96 10106 A (INNOVATIVE TECHNOLOGIES LTD.) 4 April 1996 (1996-04-04) cited in the application the whole document	32-55

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 September 2000	25/09/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Ryckebosch, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int[redacted] Application No
PCT/GB 00/00145

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 199005 Derwent Publications Ltd., London, GB; Class A96, AN 1990-032925 XP002147316 & JP 01 309682 A (SANYO CHEM IND LTD), 14 December 1989 (1989-12-14) abstract -----	1,2,18, 28-30, 32,37,62

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Application No
PCT/GB 00/00145

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9812228 A	26-03-1998	AU 4493097 A	14-04-1998
		EP 0927196 A	07-07-1999
WO 9610106 A	04-04-1996	AU 3530695 A	19-04-1996
		EP 0783605 A	16-07-1997
		GB 2307687 A,B	04-06-1997
		JP 10506442 T	23-06-1998
		US 6080420 A	27-06-2000
JP 1309682 A	14-12-1989	NONE	

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12N 5/00, A61L 15/00, 27/00, C08L 5/00

A2

(11) International Publication Number: **WO 00/49135**

(43) International Publication Date: 24 August 2000 (24.08.00)

(21) International Application Number: PCT/GB00/00145

(22) International Filing Date: 21 January 2000 (21.01.00)

(30) Priority Data:

9901272–6	21 January 1999 (21.01.99)	GB
9903561–0	17 February 1999 (17.02.99)	GB

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: CELL GROWTH

(57) Abstract

A substrate for cell growth comprises a polysaccharide and a cell adhesion protein provided at the surface of the substrate. The polysaccharide may for example be an alginate and the cell adhesion protein may be fibronectin, vitronectin or von Willebrand protein. The substrate may for example be a fibre and may be produced by extruding a solution containing dissolved polysaccharide and cell adhesion protein into a coagulation bath so that a substrate comprised of a basal layer of the polysaccharide and surface layer of cell adhesion protein is precipitated.

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CELL GROWTH

The present invention relates to substrates for use in cell growth and to methods of producing such substrates. The invention relates more particularly to substrates having cell adhesion promoting activity which may be used in various cell growth applications, e.g. wound healing and tissue engineering. The invention also relates to methods of preparing such substrates and their use in various cell growth applications.

All eukaryotic, mammalian cells are substrate dependent in that they need to be attached to a surface in order to be able to grow, or secrete or divide. The phenotype that cells express is partly determined by their interaction with the substrate to which they are attached. The substrate to which mammalian cells are attached is collagen. All body soft (excluding blood) and hard tissues are made up of cells attached to a framework of collagen. Collagen is a protein that forms fibres and the fibres form matrices, these matrices may form any configuration from random to aligned.

The collagen fibres are themselves made up of fibrils so a collagen fibre resembles a cable of aligned fibrils. The chemistry of the collagen fibril varies according to the tissue type and a range of collagens have been identified.

Substrates for tissue augmentation or to act as carriers for cultured cell transfer in wound therapy are usually collagen based. In this situation, the collagen substrate usually has to be specific to the type of cell growth required and the phenotype and status (secretory, replicatory) grown on the substrate may not turn out to be as required.

US-A-5 610 148 (R.Brown) entitled "Macroscopically Orientated Cell Adhesion Protein" describes the production of a fibre comprised of fibrils of a cell adhesion protein selected from fibronectin (Fn), vitronectin and von Willebrand protein that has been denatured and the polymer chains then aligned by unidirectional

shear allowing aggregation and precipitation. These fibres are of a fibular construction not dissimilar in some respects to collagen. Cells seeded onto the fibres demonstrate directional cell growth as a result of the longitudinal orientation of the cell adhesion binding site. However such fibre structures require a high concentration of fibronectin or fibrinogen/fibronectin, are somewhat complicated to produce and are of relatively low strength.

It is an object of the present invention obviate or mitigate the above mentioned disadvantages.

According to the present invention there is provided a substrate for cell growth comprising a polysaccharide and a cell adhesion protein provided at the surface of the substrate.

Substrates in accordance with the invention have the advantage (over substrates comprised of fibrils of fibronectin or other cell adhesion protein) of being of higher strength than a substrate comprised substantially of 100% protein and are also easier to manufacture. The substrates of the invention may be used in a wide range of cell growth applications, e.g. wound repair, tissue repair or augmentation, or for the growth of cells in routine cell culture in vitro, in large scale cell culture, bioreactors or organ culture.

In the substrates of the invention, the orientation of the cell adhesion protein is not necessarily significant and guidance of the cells during growth thereof is achieved by the physical form of the substrate. Thus, for example, in the case of a fibre (see below) cell growth may occur along and/or around the fibre as determined by the presence of the cell adhesion protein. We do not however preclude the possibility of the cell adhesion protein having at least some degree of alignment.

The cell adhesion protein preferably incorporates the RGD (Arginine, Glycuse, Aspartic acid) binding site. It is particularly preferred that the cell adhesion

protein is fibronectin, vitronectin or von Willebrand protein or a fragment of such proteins incorporating this RGD binding site.

The preferred cell adhesion protein is fibronectin which may be used in the form routinely isolated from blood plasma, e.g. by cryoprecipitation. The fibronectin may contain fibrinogen and albumin.

The polysaccharide and the cell adhesion protein may be uniformly distributed throughout the substrate so that the cell adhesion protein is present at the surface as a result of this distribution.

The substrate may comprise a polysaccharide basal layer having a surface layer of a cell adhesion protein.

The polysaccharide basal layer will for preference have a thickness of at least 60%, more preferably at least 80% and ideally at least 90% of the combined depth of the basal layer and cell adhesion protein layer.

The cell adhesion protein provided as a surface layer for the polysaccharide basal layer may be an integral layer or may be a surface absorbed molecular layer. The surface layer of the cell adhesion protein may, depending on the method by which it is produced, be only several molecules thick or may be of somewhat greater thickness so as to form a discrete outer layer. Thus, the protein layer may be anything from 3-5 molecules "deep" in the case of surface adsorption to, say, 20μm (e.g. 1-20 μm) when formed as a "coating". This protein layer may be an essentially amorphous network, have some crystallinity or even little or no fibril structure. The protein layer may be stabilised and attached to the basal (polysaccharide) layer to different degrees by different physical and/or chemical mechanisms. Examples of such attachment and stabilisation including covalent bonding, hydrogen bonding, van der Waals forces and physical entrapment. In the case where the polysaccharide incorporate carboxylic groups, covalent attachment may be achieved by a carbodiimide which "couples" a carboxylic group of the polysaccharide with an amino group of a protein. A further

possibility is the use of a melamine-formaldehyde resin. The degree of stability of the protein layer can be used as a mechanism to drive certain cell responses. Thus the substrate may be "tailored" to ensure growth of a particular cell type and/or to provide a known degree of cell growth in a predetermined time.

The polysaccharide layer will for preference comprise at least 50%, more preferably at least 60%, even more preferably at least 80% and ideally at least 90% polysaccharide. The cell adhesion protein layer will preferably comprise at least 50%, more preferably at least 60% even more preferably at least 80% and ideally at least 90% of cell adhesion protein.

The cell adhesion protein layer may incorporate proteins other than cell adhesion proteins.

Cell growth substrates in accordance with the invention may incorporate, e.g. in the polysaccharide layer, an active agent for delivery during the cell growth application. This agent may, for example, be deliverable by diffusion and might for example be a drug. Further examples of active agents include growth factors, chemotactic agents etc. The active agents may be free or encapsulated, for example in lipid type droplets. The active agent may be disposed continuously or discontinuously along, across and/or around the cell growth substrate and may be provided in different amounts at different regions of the substrate so as to establish a concentration gradient.

Substrates in accordance with the invention may be produced by a number of methods. In one such method, a solution containing dissolved polysaccharide and cell adhesion protein (the solution containing less of the protein than the polysaccharide) is extruded into a coagulation bath. We believe that, in such a method, there is preferential deposition of the cell adhesion. The coagulation bath may incorporate, for example, di- or higher- valent cations (e.g. Ca^{2+}) which serve to effect the precipitation and also stabilise the protein layer by ion bridging.

In a further method, the polysaccharide is extended into a coagulation both which incorporates protein (containing the cell adhesion protein) as the coagulant. The protein coagulant may for example be an enriched blood plasma (containing a cell adhesion protein). Once again we believe that there is preferential deposition of the cell adhesion protein at the surface of the substrate. This procedure is particularly effective when the polysaccharide is chitosan.

In an alternative method of producing the substrate, a surface layer of a cell adhesion protein may be applied to a preformed polysaccharide. Application of the protein layer may be effected, for example, in a coating bath containing a solution of protein or by a technique such as spraying. Stabilisation of the surface layer may be by a carbodiimide.

Examples of polysaccharides which may be used for the substrate include alginates, chitosan, polylysine and other polyamino acids, cationic starches, cationic derivatives of other hydrocolloids, collagen, gelatine, low-methoxyl pectin, carrageenans, chondroitin sulphate, hyaluronic acid, carboxymethyl cellulose, carboxymethyl starch, carboxymethyl guar, cellulose sulphate, dextron sulphate, gellan, xanthan, and anionic derivatives of other hydrocolloids. We particularly prefer that the polysaccharide is comprised of an alginate material cross-linked with calcium ions as other di- or higher valent cation capable of cross-linking alginates.

Particularly preferred examples of cell growth substrates in accordance with the invention are in the form of fibres having a core (providing the basal layer) which consists of, or is rich in, the polysaccharide material and a surface at which the cell adhesion protein is provided.

Fibres in accordance with the invention may have a diameter of 10-1000μm, more preferably 40-150μm, even more preferably 40-100μm, and ideally 50-80μm. the fibres may be of any appropriate length.

Such fibres may be produced by spinning a dope comprised of a solution of the polysaccharide into a coagulation bath causing precipitation of the fibres. The dope may also contain dissolved cell adhesion protein which is to form the surface layer with the spinning technique being such that there is preferential initial precipitation of polysaccharide in the coagulation bath followed by later precipitation of the cell adhesion protein which thus forms a protein rich outer layer of the fibre (this layer being integral with the core). The dope for use in this process may for example comprise (based on the total weight of the polysaccharide and cell adhesion protein) 60-95% (preferably about 90%) by weight of the polysaccharide and 5-40% (preferably about 10%) by weight of the cell adhesion protein. The fibre produced by such a process may have a core comprised of 50-80% by weight of the polysaccharide and an outer layer comprised of 50-80% by weight of the cell adhesion protein and 20-50% by weight of the polysaccharide.

In an alternative spinning method, fibres may be formed by a co-axial extrusion technique in which a solution of the cell adhesion protein is extruded co-axially around a (separate) solution of the polysaccharide, both solutions being spun into the same coagulation bath, whereby a fibre having a polysaccharide core and a surface layer of the cell adhesion protein is formed.

In an alternative process of producing the fibres, a dope comprised of a solution of the polysaccharide (but not the cell adhesion protein) may be spun into a coagulation bath and the fibre thus formed is treated with the cell adhesion protein. This treatment may be effected, for example, by providing the cell adhesion protein in the coagulation bath so that the protein is adsorbed as a surface layer onto the basal polysaccharide layer. It is however more preferred that the cell adhesion protein is applied in a bath downstream of the coagulation bath. The conditions in the protein bath may be such as to ensure formation of a stabilised coating of the protein layer is obtained.

Furthermore, for all embodiments of fibre formation, the cell adhesion protein should be concentrated at the fibre surface. If the fibre is produced by co-spinning a

solution of the polysaccharide and cell adhesion protein the combination of relative molecular size, hydrophilic/hydrophobic balance and relative stability can be used to cause preferential precipitation If the fibre is produced by a two-stage process then concentration of the protein at the surface may be achieved by the use of concentration of the polysaccharide and protein at each stage, first stage mixed polysaccharide and protein, second stage predominantly protein plus surface active agents and/or stabilisers.

Whichever method is used, the protein should be stabilised at the surface and, in fact, the lower the amount of protein the more important the stabilisation becomes. Stabilisation may be effected by ensuring that parts of the molecular chain of the protein are embedded in the bulk polysaccharide. In the case where the polysaccharide has been cross-linked by divalent cations, stabilisation of the protein may be by divalent cation bridges. When chitosan is used to form the core, carrier cation bridging will only occur within the protein species which will help to stabilise the protein at the surface.

More specific embodiments of producing fibres in accordance with the invention are described below.

In one such embodiment, a fibre is produced by ejecting an aqueous solution of sodium alginate through a spinneret into a coagulation both containing Ca^{2-} ions. The fibre is then passed through a fibronectin solution (or mixed protein solution) in a coating bath (downstream of the coagulation bath) which is at a pH that will give fibronectin a net positive charge causing it to be capable of interacting with the alginic acid. The fibronectin can be further bound to the alginate by passing the fibre through a coagulation/stabilisation bath at a pH that favours fibronectin to become negatively charged thus favouring divalent cation bridging so as to stabilise the fibronectin on the polysaccharide. Alternatively, this bath may incorporate carbodiimide for effecting covalent bonding if the protein to the polysaccharide the coagulation/stabilisation bath may contain agents that modify either directly the

interaction of the fibre with cells (for example through the nature of a counterion, e.g. Zm, Ag, Mn, Ce) or indirectly by influencing the surrounding environment by diffusion of an active molecular species, such as growth factors, aggregating agents, chemoattractants, surfactants, etc.

As an alternative to applying the fibronectin in a coating bath, it is possible to apply a fibronectin coating by spraying a fibronectin solution onto the fibre. Spaying provides. Spraying provides a means of thin coating (i.e. only several molecules thick) and also a method of coating that will potentially produce a fibrillar form of the coating if the conditions of shear etc. are set correctly. These conditions may also be adjusted to give orientation of the fibril formed in relation to the substrate.

In a further embodiment of fibre production, fibres may be found in a single stage process by spinning a dope containing dissolved sodium alginate and fibronection into a solution of calcium or other divalent ions (which provide the driving force for precipitation). The dope is formulated such that the fibronectin is preferentially precipitated at the surface of the fibre. The relative amounts of the calcium alginate to the fibronectin in the dope would preferably be of the order of at least 80 parts by weight alginate and at most 20 parts fibronectin.

In the process described in the preceding paragraph, the fibre would be produced under conditions that encourage the globular nature of the protein. This may be achieved by use of a pH or temperature (for the coagulation bath) that causes chains of the protein molecule to "roll-up" on themselves with a tendency to embed the ends of the chain in the fibre structure.

In an alternative fibre production the process, a polyelectrolyte such as chitosan would be mixed with the fibronectin solution and a fibre precipitated by spinning into a sodium hydroxide bath. The molecular weight of the chitosan would be chosen to encourage fibre formation.

As an alternative to the process described in the previous paragraph it is possible to spin a dope comprising a solution of chitosan (as the polysaccharide) to form a fibre which may subsequently be coated with fibronectin. This coating (of fibronectin) would be formed by charge interaction directly between the charged chitosan side chains and the amino acid groups of the fibronectin as well as by cationic bridging.

For all methods of fibre production, it may be appropriate to subject the spun fibres to stretching, washing, and/or drying operations. In the case where a (separate) surface treatment of the cell adhesion protein is applied after formation of the basal polysaccharide layer, it may be appropriate to effect stretching and/or washing prior to the treatment with the cell adhesion protein.

Whilst fibres are the preferred form of the cell growth substrate in accordance with the invention, other forms are possible. Examples include sheets and strips which may be produced by forming (by a knife over roll or transfer coat or slot dye method) a thin film of a solution of the polysaccharide which is then precipitated in a coagulation bath. As in the case of fibre formation, the solution may also incorporate the cell adhesion protein to be preferentially deposited on coagulation at the surface of the polysaccharide. Alternatively the solution to be precipitated in the coagulation bath need not include the cell adhesion protein which may then be applied subsequently to the sheet or strip by spraying with a solution of the protein. In this case, the nature of the coating is determined by the concentration of the protein in solution, the velocity, orifice, size and direction of spray relative to the surface. Judicious adjustment of these parameters should produce undenatured but aligned molecules of active protein. The surface layer of the cell adhesion protein may be applied to the sheet by spraying with a solution of the protein. By spraying at high concentration and flow rate through a small orifice, protein denaturation, fibril formation and alignment can be obtained and if this is directed in parallel to a surface then this alignment will be maintained in the surface coat obtained molecular alignment of the protein will then be reflected in the alignment of cellular species grown on the substrate.

Irrespective of the physical form (fibre, sheet etc) of the cell growth substrate of the invention and also irrespective of the manner in which the cell adhesion protein surface layer is incorporated therein, it is preferred that basal polysaccharide layer is formed by a spinning or extruding a solution of sodium alginate into a bath containing calcium ions. Preferred sodium alginate for use in such a technique have a Guluronic acid (G) content of at least 35% by weight and a Mannuronic acid (M) content of at most 65% by weight. Preferably the G-content is 35-70% by weight and the M-content is 65-30% by weight. M preferably also the sodium alginate has a viscosity for a 1% solution (in water) of the sodium alginate of 30-300 cP, more preferably 40-100 cP. The alginate solution to be spun or extruded into the coagulation bath should generally have a total dissolved solids content of less than 10% by weight, more preferably in the range 5-7%. The amount of the cation (e.g. calcium) present in the coagulation bath (to effect precipitation of the alginate) is preferably less than 1% by weight.

For products in accordance with the invention produced by coagulation of a solution of an alginate, it is possible for the alginate solution (to be coagulated) to contain at least one additional polysaccharide to modify the properties of the alginate. The additional polysaccharide may, for example, be one having COO^- groups along the polysaccharide chains, for example pectin, carboxymethyl cellulose N-, O-carboxymethyl chitosan, carrageenan, xanthan or gellan. Alternatively or additionally the polysaccharide to the coagulated with the alginate may be one having SO_4^{2-} groups provided along the polysaccharide chain, e.g. chondroitin sulphate, dermatan sulphate, heparan sulphate or heparan. Uncharged polysaccharides may be used in conjunction with the alginate, e.g. acemannan. The additional polysaccharide may be one which improves the water absorbency of the alginate. Further disclosure of products obtained by coagulation of an alginate solution containing at least one other polysaccharide are given in WO-A-9610106 (Innovative Technologies Ltd), the disclosure of which is incorporated herein by reference.

For all cell growth substrates in accordance with the invention, the surface layer of the cell adhesion protein may be continuous or discontinuous. Thus, for example, in the case of a fibre, the protein may be provided continuously along and around the fibre length or as periodic repeats (e.g. of predetermined length) along the fibre length and at least partially around the circumference of the fibre, or as "stripe" which does not extend completely around the circumference and which extends continuously or discontinuously along the fibre length. If the cell adhesion protein layer is discontinuous, parts of the surface of the cell growth substrate may (when used for cell growth) be positively interactive ("talking") and other parts passive ("silent") and other parts negatively interactive ("discouraging"). In cellular terms, this means that a positive surface encourages cell adhesion spreading, motility and growth whereas a passive surface ("silent") may have a low level of interaction

Cell growth substrates in accordance with the invention may be used in a number of forms for various cell growth applications. Purely by way of example, substrates in the form of fibres may be formed into a structure, e.g. random matrices (e.g. non-woven felts and fleeces), orientated matrices (fibres having some relative alignment), knitted structures (e.g. knitted cloths), braided structures (e.g. braided thread), bundled structures, and carded slivers. One preferred structure comprises fibres in accordance with invention laid in parallel or randomly to each other and for preference bonded to a supporting layer, e.g. a polyurethane film. This supporting layer may be adhesively coated.

A further possibility is for fibres to be arranged in an amorphous gel.

A further possibility relates to fibres produced with a polysaccharide (e.g. alginate) cross-linked by a di- or higher-valent cation (e.g. calcium). Such fibres may (using the techniques disclosed in WO-A-9613285 (Innovative Technologies Ltd) be admixed with an aqueous solution of a hydrogel precursor material whereby the cations from the fibres cross-link the precursor material resulting in the formation of a hydrogel in which the molecules of the hydrogel precursor are cross-linked by the di- or higher-valent cations donated by the fibres. The admixture may incorporate a plasticiser. Subsequently water may be removed from the hydrogel so as to provide a

dehydrated form thereof containing the fibres as reinforcement. Such a product is eminently suitable for use on wound healing during which fibres will become exposed at the surface of the product to provide a substrate for cell growth. The hydrogel precursor may for example be sodium alginate and the plasticiser may for example be glycerol, polyethylene glycol, sorbitol or a PEO/PPO polymer.

Cell growth substrates in the form of strips or sheets may for example be rolled into tubes or other three dimensional structures.

As indicated above, cell growth substrates in accordance with the invention may be used in a range of cell growth applications. If cell alignment on the surface of the substrate is important then this may be imposed by the nature of the cell and its relationship to its surface. For example, cell alignment may be determined by the size of a fibre on which the cell is grown. If cell-long alignment either across or parallel to a particular axis of the substrate is required then this can be accomplished by either exposure of the surface to flow which will produce a wall shear stress parallel to the desired orientation or to axial strain which would tend to cause the cells to lie across the axis of stress and therefore across the axis of the surface.

A number of specific (but non-limiting) example of uses of cell growth substrates in accordance with the invention will now be given.

Wound Therapy

The substrates may be used in wound therapy. For this purpose, a cell-growth substrate (in accordance with the invention) in the form of a flat sheet or film may be preferred. The film or sheet material may incorporate an agent to be delivered to the wound.

Alternatively, parallel or random arrays of fibres with or without seeded cells may be placed on the wound either individually, in a bundled or fixed to a support which may be adhesively coated. An example of a suitable support is polymeric film material particularly a breathable film (e.g. high MVTR film). The film may be one having an MVTR when in contact with liquid water which is at least twice that when

in contact with moisture vapour (but not liquid water). For example, the MVTR in contact with water vapour only may be 3000-5000 g m^{-2} $24hr^{-1}$ (as measured by ASTM E96B) and an MVTR in the presence of liquid water (as measured by ASTM E96BW) of 8000 to 10000 g m^{-2} $24hr^{-1}$. The support may have apparatus to allow exudate transfer. Whether or not a support is used, the fibres applied to the wound may incorporate growth factors for delivery to surface cells or incorporate agents that will influence the surrounding environment, e.g. bactericides etc. Mixtures of fibres may be applied to the wound, e.g. any two of (i) fibres seeded with cells, (ii) unseeded fibres, and (iii) fibres containing an agent to be delivered to the wound.

Cultured Epidermal and Dermal Substitutes

Cell growth substrates in accordance with the invention may be cultured with single layers of epidermal keratinocytes or dermal fibrobalasts (either of which may be of autologous or heterologous origin.) The substrate (with cultured cells) may be used alone or in combination with similarly cultured substrates. These substrates and cells may be used for the treatment of partial thickness wounds, e.g. donor sites and for treatment of ulcers.

Tissue Augmentation/Repair

Cell growth substrates in the form of continuous fibres can be positioned in relation to a damaged organ or structure. They may be placed either singularly or in bundles during invasive or non-invasive therapy.

Alternatively, cell growth substrates in the form of fibres may be provided as an injectable suspension. The suspension may be introduced into the body along a catheter guide system or the fibres may be formed at the site. As an alternative, it is possible to formulate to solution one containing the polysaccharide, the other coagulant therefore with at least one of the solutions containing cell adhesion protein and to apply these solutions to a patient under conditions such that fibre formation occurs *in situ*, the fibre formed possibly being continuous.

Orthopaedic

Cell growth substrates in the form of fibres may be aligned parallel to tendons and seeded *in situ* with appropriate cells, chondrocytes, etc. Alternatively, fibres plus cell may be cultured in a laboratory and then delivered to the patient. For both embodiments, the fibres may contain, or be associated with fibres constructed with hyaluronic acid or other cartilage-derived substances.

Vascular Graft

Cell growth substrates in the form of fibres may be knitted, woven or spun into tubes to encourage cell growth to form a blood conduit.

Nerve Regeneration

Damaged nerves can be repaired using fibres to link the two (separated) ends of the nerve thus providing a path along which the new nerve can grow.

Drug delivery

Cell growth substrates may incorporate active molecules located in the polysaccharide layer. These agents may be used to influence the fibre incorporation into the tissue. Alternatively the agent may provide a drug reservoir for the purposes topical or systemic therapy.

For all of the above embodiments of the invention, the cell adhesion protein may be replaced by a blood plasma component.

The invention is further illustrated with reference to the following non-linking Examples and the Figures of the accompanying drawings which shown the results of the Examples.

For the Examples, the following procedures were used.

Cell Culture

L929 mouse fibroblast cells for use in an experiment were grown to confluence and then released from the tissue culture dishes by washing with Hepes Saline, followed by treatment with 0.25% trypsin solution. The resulting supernatant was centrifuged and the pellet of cells re-suspended in Dulbecco's modified Eagle's Medium [containing 10% Foetal calf serum, 5% Penicillin/Streptomyocin, 1% ITS (Insulin transferrin selenite)]. If being sub-cultured, then the cells were plated out on tissue culture plates at a 1:5 dilution.

15mg of each fibre type to be tested were weighed out and placed in each well of a 12 well tissue culture dish. In all experiments the fibres were washed in serum containing media for a period of 24 hours. The experimental controls were cells plated on tissue culture plastic. Cells used for fibre testing were plated out at a density of 80,000 cells per well. All experiments were terminated up to a 72 hour timepoint.

Fixation and Staining

The cells and fibres were washed twice in Phosphate Buffered Saline (PBS) and then fixed using formalin solution (10% neutral buffered) for 10 minutes. The fixative was removed and the cells and fibres washed twice more in PBS. The cells were then stained with Geimsa for 10 minutes, followed by 3, five minute washes in PBS. The cells were then viewed using a Nikon Diaphot microscope and images captured using a JVC DV1 digital camcorder. The images were then downloaded to an Apple Macintosh Power PC Performa 6400/200 and analysis performed using the public domain program NIH image. For the scanning electron microscopy, fixed samples were dehydrated in 100% ethanol for a period of 2 hours. The samples were then sputter coated using a Denton Vacuum desk 1. The samples were mounted on a stub and viewed using a Hitachi S-510 scanning electron microscope. Images are captured using the JVC camera and analysed on the Macintosh computer using NIH image.

Preparation of Enriched Bovine Blood Plasma

Bovine blood was taken and mixed in a 9:1 ratio with a 4%w/w aqueous solution of trisodium citrate (Sigma Chemicals) as an anti-coagulant. The mixture was then centrifuged at 1000rpm for 10 minutes, after which time the supernatant plasma was pipetted off, frozen at −15 to 20 °C and then thawed under refrigeration at 4°C. This caused the globular protein content of the plasma to remain precipitated and become concentrated by sedimentation at the bottom of the storage vessel. The supernatant from the refrigerated plasma was removed and the remaining fraction when thawed formed a plasma further enriched in globular protein concentration which may be further enriched by another freeze thaw cycle. The plasma fraction thus isolated was used in some of the experiments outlined in the Examples below.

Example 1 (Comparative)

Calcium alginate fibres were produced by ejection at 12m/min of a 5.5%w/w aqueous solution of sodium alginate (ex Pronova Biopolymer, having a guluronic acid content of 70%) through a spinneret having 40,000 holes each of 70μ diameter into a coagulation bath of calcium chloride dihydrate (1.5%w/w) and the resulting fibres were washed in acetone and dried. The fibres were observed under a scanning electron microscope (Hitachi model S510) and were found to be about 10-20μ diameter smooth, cylindrical with few outstanding surface topographical features (see Figure 1). The cell adhesion properties of the fibres were then assessed using L929 mouse fibroblast cells according to the method outlined above. No cell attachment to the fibres was observed within 2 hours during which time the fibres formed a gel and then disintegrated.

Example 2

A mixture of 5.5%w/w aqueous solution of sodium alginate (as in Example 1) with bovine blood plasma was prepared by mixing the components in a ratio of 3:2. This mixture was then used to produce fibres in the laboratory by ejection from a 1ml insulin syringe through a needle of 35μ outside diameter into a coagulation bath of calcium chloride dihydrate (1.5%w/w) and the resulting fibres were washed in acetone and dried. Fibres were observed under the scanning electron microscope (see Figure 2a). The cell adhesion properties of the fibres were then assessed using L929 mouse fibroblast cells according to the method outlined above. Within 48 hours cells had grown to confluence on the fibres (see Figure 2b), a considerable improvement of the result observed in Example 1.

Example 3 (Comparative)

A 3% w/w of chitosan, having a degree of de-acetylation >70% (available from Nigerian Fisheries), in 2% aqueous glacial acetic acid was prepared.

Chitosan fibres were made in the laboratory by ejecting the chitosan solution from a 1ml insulin syringe through a needle of 35μ outside diameter into a coagulation bath of sodium hydroxide (5%w/w) and the resulting fibres were dried. The fibres were observed under the scanning electron microscope, the fibres were found to have a diameter of 40-100μ and to be smooth, cylindrical with few outstanding surface topographical features. The cell adhesion properties of the fibres were then assessed using L929 mouse fibroblast cells according to the method outlined above. After 48 hours, a number of cells were found to adhere to the fibres but no evidence for cell elongation and alignment was apparent (see Figure 3).

Example 4

Chitosan fibres were made in the laboratory by ejecting a solution of chitosan (as specified in Example 3) from a 1ml insulin syringe through a needle of 35μ outside diameter into a coagulation bath of enriched bovine blood plasma (isolated as described above) and the resulting fibres were washed in acetone and dried. The cell adhesion properties of the fibres were then assessed using L929 mouse fibroblast cells according to the method outlined above. Within 48 hours cells h ad grown to confluence (as seen from Figure 4) on the fibres, a far higher degree of cell attachment than that observed for fibres coagulated in sodium hydroxide (compare Example 3).

Claims

1. A substrate for cell growth comprising a polysaccharide and a cell adhesion protein provided at the surface of the substrate.

2. A substrate as claimed in claim 1 comprising a polysaccharide basal layer having a surface layer incorporating a cell adhesion protein.

3. A substrate as claimed in claim 2 wherein the polysaccharide basal layer has a thickness greater than 60% of the thickness of this layer and the polysaccharide basal layer.

4. A substrate as claimed in claim 3 wherein the polysaccharide basal layer has a thickness greater than 80% of the thickness of this layer and the polysaccharide basal layer.

5. A substrate as claimed in any one of claims 2 to 4 wherein the cell adhesion protein layer is integral with the polysaccharide basal layer.

6. A substrate as claimed in claim 5 wherein the layer of the cell adhesion protein has a thickness of 1-20μm.

7. A substrate as claimed in any one of claims 2 to 5 wherein the layer of the cell adhesion protein is a surface adsorbed layer.

8. A substrate as claimed in claim 7 wherein the layer of the cell adhesion protein is 3-5 molecules deep.

9. A substrate as claimed in any one of claims 2 to 8 wherein the polysaccharide basal layer comprises at least 80% by weight of polysaccharide.

10. A substrate as claimed in claim 9 wherein the polysaccharide basal layer comprises at least 90% by weight of polysaccharide.

11. A substrate as claimed in any one of claims 2 to 10 wherein the surface layer of the cell adhesion protein comprises at least 80% by weight of cell adhesion protein.

12. A substrate as claimed in claim 11 wherein the surface layer of the cell adhesion protein comprises at least 90% by weight of cell adhesion protein.

13. A substrate as claimed in claim 12 wherein the surface layer of cell adhesion protein comprises 95 to 100% by weight of cell adhesion protein.

14. A substrate as claimed in any one of claims 2 to 13 wherein the cell adhesion protein layer is discontinuous layer.

15. A substrate as claimed in any one of claims 2 to 14 wherein the polysaccharide layer incorporates an active agent.

16. A substrate as claimed in claim 15 wherein the active agent is encapsulated.

17. A substrate as claimed in claim 15 or 16 wherein the active agent is a drug, growth factor or chemotactic agent.

18. A substrate as claimed in any one of claims 1 to 17 wherein the polysaccharide is selected from alginates, chitosan, polylysine and other polyamino acids, cationic starches, cationic derivatives of other hydrocolloids, collagen, gelatine, low-methoxyl pectin, carrageenans, chondroitin sulphate, hyaluronic acid, carboxymethyl cellulose, carboxymethyl starch, carboxymethyl guar, cellulose sulphate, dextran sulphate, gellan, xanthan, and anionic derivatives of other hydrocolloids.

19. A substrate as claimed in claim 18 wherein the polysaccharide is an alginate.

20. A substrate as claimed in claim 19 wherein the alginate has a Guluronic acid (G) content of at least 35% by weight and Mannuronic acid (M) content of at most 65% by weight.

21. A substrate as claimed in claim 20 wherein the polysaccharide as a G content of 35-70% by weight and an M content of 65-30% by weight.

22. A substrate as claimed in any one of claims 19 to 21 wherein the alginate is cross-linked with divalent cations, preferably calcium ions.

23. A substrate as claimed in claim 22 wherein the cell adhesion protein is stabilised by calcium ion bridges.

24. A substrate as claimed in claim 18 wherein the polysaccharide is chitosan.

25. A substrate as claimed in claim 24 wherein the chitosan comprises at least 70% de-acetylated chitin.

26. A substrate as claimed in any one of claims 1 to 24 wherein the cell adhesion protein is stabilised by carbodiimide.

27. A substrate as claimed in any one of claims 1 to 26 wherein the cell adhesion protein is present in blood plasma.

28. A substrate as claimed in any one of claims 1 to 27 wherein the cell adhesion protein incorporates the RGD binding site.

29. A substrate as claimed in claim 28 wherein the cell adhesion protein is fibronectin, vitronectin or von Willebrand protein.

30. A substrate as claimed in claim 29 wherein the cell adhesion protein is fibronectin.

31. A substrate for cell growth comprising a polysaccharide and a blood plasma component provided at the surface of the substrate.

32. A substrate as claimed in any one of claims 1 to 31 in the form of a fibre.

33. A substrate as claimed in claim 32 wherein the fibre has a diameter of 10-1000μm.

34. A substrate as claimed in claim 33 wherein the fibre has a diameter of 40-150μm.

35. A substrate as claimed in claim 34 wherein the fibre has a diameter of 40-100μm.

36. A substrate as claimed in claim 35 wherein the fibre has a diameter of 50-80μm.

37. A substrate as claimed in any one of claims 1 to 31 which is in the form of a sheet or film.

38. A substrate as claimed in claim 37 having a thickness of 2-2000μm.

39. A substrate as claimed in claim 38 having a thickness of 10-100μm.

40. A substrate as claimed in claim 38 having a thickness of 200-1000μm

41. A substrate as claimed in claim 38 having a thickness of 500-2000μm.

42. An assembly of fibres as claimed in any one of claims 32 to 36.

43. An assembly as claimed in claim 42 in the form of a random matrix (e.g. a non-woven felt or fleece), orientated matrix (fibres having some relative alignment), a knitted structure, a braided structure, a bundled structure or a carded sliver.

44. An assembly comprising a plurality of fibres as claimed in any one of claims 32 or 36 wherein the fibres are arranged in parallel to each other.

45. An assembly comprising a plurality of fibres as claimed in any one of claims32 or 35 wherein the fibres are arranged randomly.

46. An assembly as claimed in claim 44 or 45 wherein the fibres are provided on a support in the form of a sheet or film.

47. An assembly as claimed in claim 46 wherein the fibres are provided on a high MVTR film.

48. An assembly as claimed in claim 42 wherein said fibres are provided in a matrix of an amorphous gel.

49. A method of producing a cell growth substrate comprising extruding a solution containing dissolved polysaccharide and cell adhesion protein, the polysaccharide being present in an amount greater than the cell adhesion protein, into a coagulation bath such that a substrate comprised of a basal layer of a polysaccharide and a surface layer of cell adhesion protein is precipitated.

50. A method as claimed in claim 49 wherein the substrate is precipitated in a bath containing divalent cations typically calcium ions.

51. A method as claimed in claim 49 or 50 wherein the solution to be extruded comprises (based on the total weight of the polysaccharide and cell adhesion protein) 60-99% by weight of the polysaccharide and 1-40% by weight of the cell adhesion protein.

52. A method as claimed in any one of claims 50 to 51 wherein the dissolved polysaccharide is sodium alginate.

53. A method as claimed in claim 52 wherein the sodium alginate has a G-content of 35-70% by weight and an M-content of 65-35% by weight.

54. A method as claimed in claim 52 or 53 wherein the sodium alginate has a viscosity for a 1% solution (in water) of 30-300 cP.

55. A method as claimed in claim 54 wherein the sodium alginate has a viscosity of a 1% solution (in water) of 40-100 cP.

56. A method of producing a cell growth substrate comprising applying to the surface of a preformed layer of a polysaccharide a surface layer of a cell adhesion protein or blood plasma component.

57. A method as claimed in claim 54 wherein the method of application is by immersion of the polysaccharide layer in a coating bath containing the cell adhesion protein or blood plasma component.

58. A method as claimed in claim 54 wherein the polysaccharide is precipitated into a coagulation bath containing the cell adhesion protein.

59. A method as claimed in claim 56 wherein the method of applications by spraying.

60. A method as claimed in any one of claims 56 to 59 effected with stabilisation of the protein layer.

61. A method as claimed in claim 60 wherein the stabilisation is effected with carbodiimide.

62. A method of cell culture comprising effecting growth of cells on a substrate as claimed in any one of claims 1 to 49 or an assembly as claimed in any one of claims 42 to 48.

63. A method as claimed in 62 wherein the cell growth is for wound repair, tissue repair or augmentation, culturing epidermal or dermal substitute, orthopaedic application, vascular grafts, nerve regeneration or cell culture in a bioreactor.

64. The use of a substrate as claimed in any one of claims 1 to 49 or an assembly as claimed in any one of claims 42 to 48 in therapy.

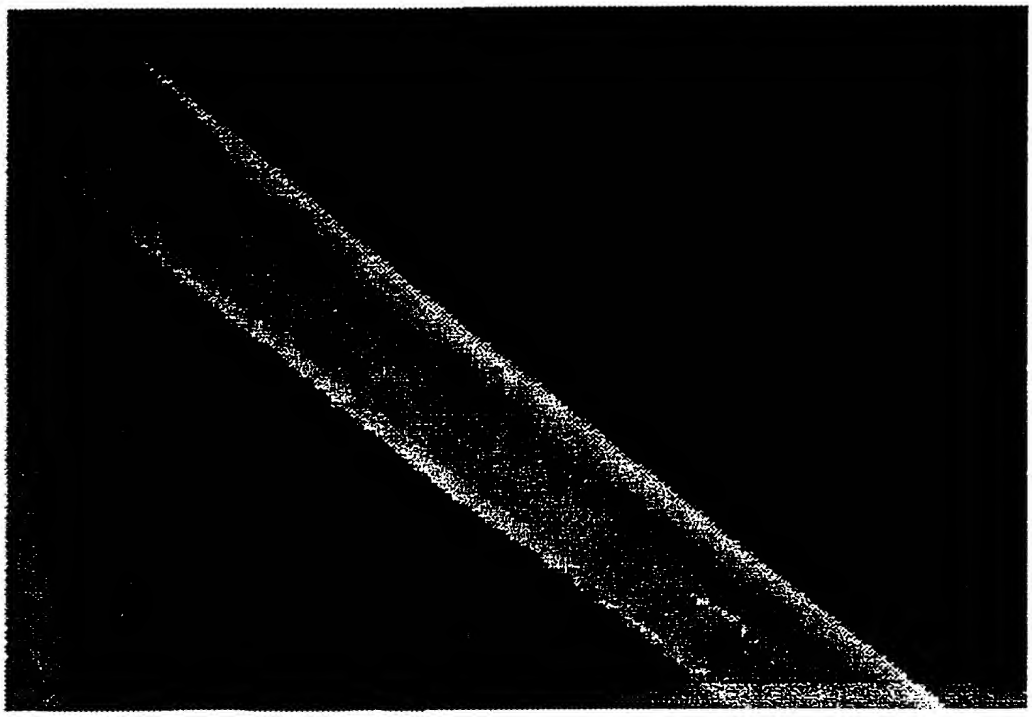

FIG. 1

FIG. 2a

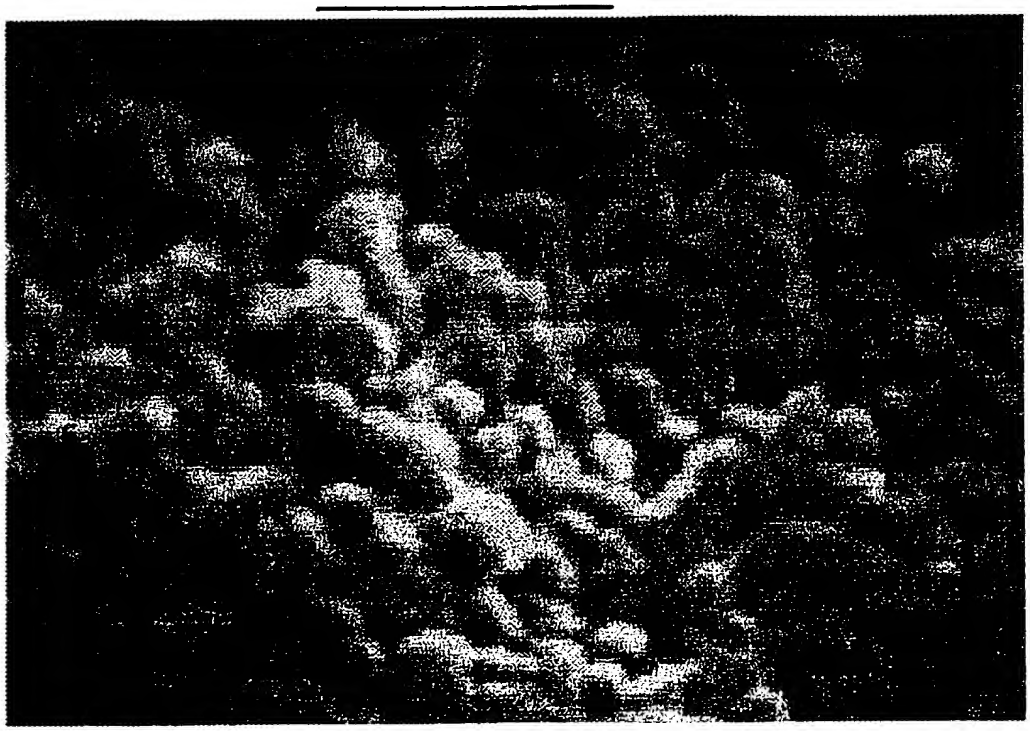

FIG. 2b

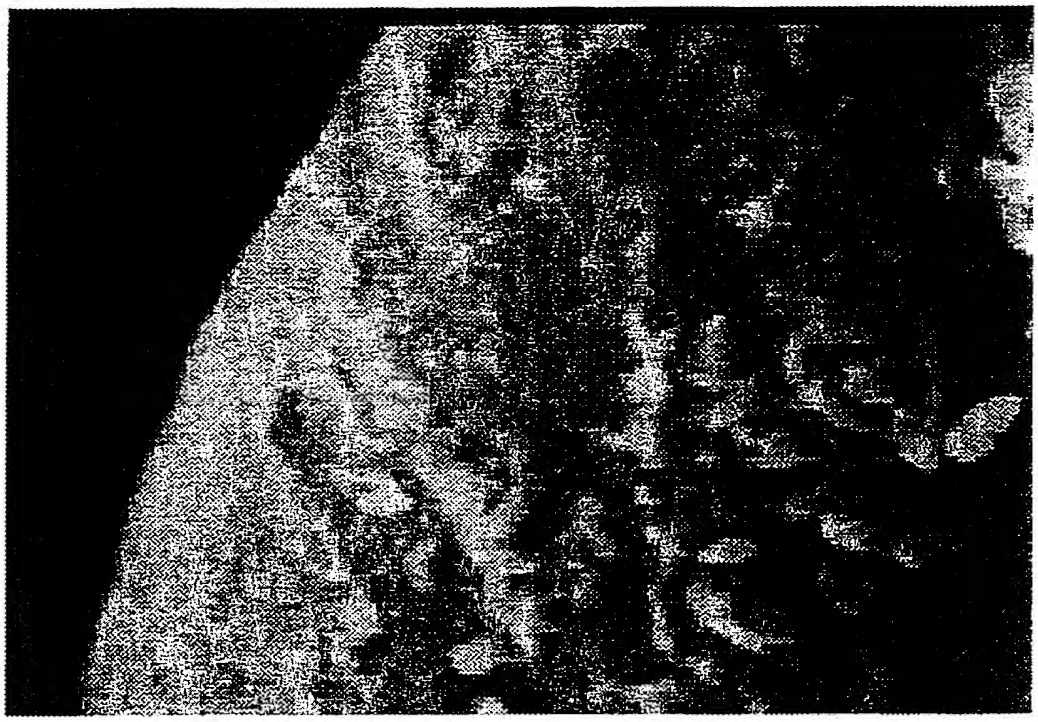

FIG. 3

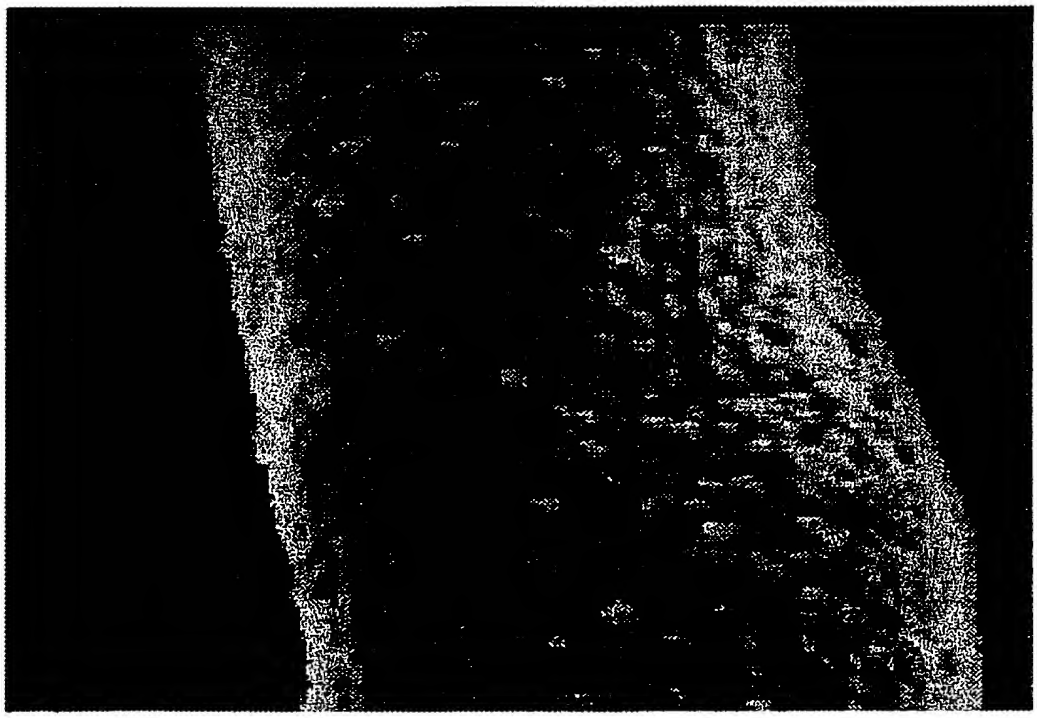

FIG. 4

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M. O.

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing *(day/month/year)*	10.04.2001
Applicant's or agent's file reference PBA/D088342PWO	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/00145	21/01/2000	21/01/1999

Applicant
ADVANCED MEDICAL SOLUTIONS LIMITED et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PBA/D088342PWO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/00145	21/01/2000	21/01/1999

International Patent Classification (IPC) or national classification and IPC
C12N5/00

Applicant
ADVANCED MEDICAL SOLUTIONS LIMITED et al.

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 7 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☐ Priority
   III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☐ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☐ Certain documents cited
   VII ☐ Certain defects in the International application
   VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
17/08/2000	10.04.2001
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Stolz, B Telephone No. +49 89 2399 8416

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/00145

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-18	as originally filed				
Claims, No.:					
1-62	as received on	16/03/2001	with letter of	16/03/2001	
Drawings, sheets:					
1/3-3/3	as originally filed				

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3),

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/00145

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-62
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-62
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-62
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

INTERNATIONAL PRELIMINARY EXAMINATION REPORT - SEPARATE SHEET

International application No. PCT/GB00/00145

1. Reasoned statement

1.1. The application describes cell growth substrates comprising a polysaccharide and a cell adhesion protein non-covalently attached thereto. The substrates can be spun into fibers or produced as sheets. They are useful for cell growth in vitro or for wound healing and tissue engineering.

1.2. The new set of claims is acceptable under Art. 34(2)(b) PCT.

1.3. Novelty (Art. 33(2) PCT)

Methods of producing polymeric substrates comprising polysaccharides and a non-covalently attached cell adhesion molecule have not been described in the cited prior art. The claimed methods and products are therefore novel.

1.4. Inventive step (Art. 33(3) PCT)

The closest prior art to the present application is WO98/12228 (D1), describing the use of a polymeric substrate such as alginate having covalently bound cell adhesion molecules. D1 described the limited usefulness of alginate hydrogels due to their lack of inherent cell adhesion (p. 5, lines 5-10). The solution to this problem provided by D1 is covalent attachment of cell adhesion molecules. The present application deals with the same technical problem but provides a different solution, i.e. non-covalent association of cell adhesion molecules with the polysaccharide substrate. Since the cited prior art does not mention non-covalent attachment of cell adhesion molecules, the claimed solution to the above mentioned technical problem cannot be said to be derivable from the prior art in an obvious way. The claims are therefore considered to be inventive.

2. Certain observations

2.1. Claim 18 lists suitable polysaccharides. Among these are also polyamino acids such as polylysine and the protein collagen (gelatin).